CONDITIONAL GENE EXPRESSION USING RNAi

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application claims priority to USSN 60/413,794, filed September 26, 2002, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with government support under Grant No. NIH 5RO1AG11816, awarded by the NIH. The government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The present invention relates to regulation of gene expression and conditional gene expression by regulating an RNAi response. More particularly, the present invention is directed to methods of inhibiting an RNAi response by contacting a cell with a dsRNA directed against a component of the RNAi response, *e.g.*, with a dcr-1 dsRNA, thereby inhibiting an RNAi response. The present invention also relates to regulation of lifespan in eukaryotes, *e.g.*, enhancement of adult lifespan via modulation of aging associated proteins; as well as to the use of expression profiles, promoters, reporter genes, markers, and compositions in diagnosis and therapy related to lifespan extension, life expectancy, and aging.

BACKGROUND OF THE INVENTION

[0004] It has been shown that introduction of small interfering RNAs (siRNAs) into a cell can cause a specific interference of gene expression known as RNA interference (RNAi). McManus *et al.*, *J. Immunol.* 169(10):5754-60 (2002). RNA interference (RNAi) is a sequence specific nucleic acid degradation mechanism triggered by double stranded RNA (dsRNA). (McManus and Sharp, *Nature Rev. Genet.* 3:737 (2002)). During RNA interference (RNAi), long dsRNA is processed into biologically active "short-interfering RNA" (siRNA) sequences of about 21-23 nucleotides in length. (Elbashir, *et al.*, Genes Dev. 15:188 (2001) and McManus *et al.*, *RNA* 8(6):842-50 (2002)). The siRNA sequences induce

degradation of complementary target single stranded mRNA and thus "silence" the corresponding translated sequences. (Elbashir, *et al.*, Genes Dev. 15:188 (2001), McManus and Sharp, *Nature Rev. Genet.* 3:737 (2002), and McManus *et al.*, *RNA* 8(6):842-50 (2002)).

[0005] The use of dsRNA interference to inhibit gene function is useful in many organisms, as it is a highly specific and robust method of inactivating gene function by causing destruction of the corresponding RNA. RNAi is therefore a powerful therapeutic tool for regulating gene expression. Destruction of the mRNA is achieved by introducing the corresponding dsRNA into the target cell or organism. Once activated, the RNAi response is extremely stable and can be heritable. Therefore, an important factor for use of this technology as a therapeutic is the ability to turn off the RNAi response once it has been established.

[0006] Many diseases and disorders, including aging disorders and disorders associated with aging and increased age are associated with abnormal expression of particular genes. Therefore, there is a need in the art for additional methods of specifically modulating gene function. The present invention solves these and other problems.

SUMMARY OF THE INVENTION

[0007] An existing RNAi response can be turned off by inhibiting genes required for the RNAi response. To turn off an existing RNAi response, a *dcr-1* (DICER) dsRNA has been introduced into an organism (*see, e.g.*, Ruvkun, *Science* 294;797 (2001)). Dicer is a key component of the RNAi machinery and loss of its function blocks the RNAi response . The use of dicer dsRNA therefore has wide applications as a modulator of the RNAi response, and provides a means for conditional gene expression using RNAi. Other genes involved in the RNAi response, such as *rde-1*, *smg-5*, *ego-1*, and *rde-4*, can also be used to block the RNAi response (*see, e.g.*, Tijsterman *et al.*, *Science* 295:694-697 (2002); Ketting *et al.*, *Genes Dev.* 15:2654-2659 (2001); Plasterk & Ketting, *Curr. Opin. Genet. Dev.* 10:562-567 (2000); Ketting *et all, Cell* 99:133-141 (19

[0008] In one embodiment, the present invention provides a method for inhibiting an RNAi response in a cell. The cell is contacted with a dsRNA involved in the RNAi response, thereby inhibiting an RNAi response in a cell. In some embodiments, the dsRNA is a dcr-1 dsRNA (*e.g.*, a human dcr-1 or a *C. elegans* dcr-1). In some embodiment, inhibiting the RNAi response in a cell modulates an age-associated parameter such as, for example,

lifespan. Modulation includes, for example, inhibition of aging or extension of lifespan. In some embodiments, inhibiting the RNAi response modulates the expression of a cellular stress-response gene, an antimicrobial gene, a metabolic gene, a steroid or lipid-soluble hormone synthesis gene, a fatty acid desaturation gene or a homolog or ortholog thereof. In some embodiments, inhibiting the RNAi response modulates the expression of a cytochrome P450, an estradiol-17-β-dehydrogenase, a alcohol/short-chain dehydrogenase, an esterase, a UDP-glucuronosyltransferase, an aminopeptidase, a carboxypeptidase, an amino-oxidase, an aminoacylase, an oligopeptide transporter, metallothionein, a receptor guanylate cyclase, a mitochondrial superoxide dismutase, a catalase, lysosyme, saposin, vitellogenin, glutathione-S-transferase, heat-shock protein, heat shock factor, an F-box/cullin/Skp protein, an isocitrate lyase, a malate synthase ASMTL, insulin, IFG1 or IFG2 or a homolog or ortholog thereof. In some embodiments, the homologs or orthologs are human homologs or orthologs.

[0009] In some embodiments, the cell is present in a subject. In one embodiment, the subject is *C. elegans*. In another embodiment, the subject is a plant. In further embodiment, the subject is a mammalian subject, *e.g.*, a mouse, a rat, a guinea pig, a monkey, or a human. In some embodiments, the mammal is not at risk for, or does not have a premature aging disorder. In some embodiments, the mammal is a post-natal mammal, *e.g.*, a mammal that has not reached sexual maturity, or an adult, or a post-reproductive adult. In an exemplary embodiment, the mammal may be a non-diabetic, non-obese adult, a healthy adult, or an adult with an abnormal aging disorder. In another exemplary embodiment, the subject can be a human that has not reached 10, 20, or 50 years of age. Or the subject can be a human that has attained at least 20, 40, 50, 70, 80, or 90 years of age.

[0010] In another embodiments, the present invention provides a method of increasing lifespan, treating premature aging, or altering lifespan regulation in a subject. A dsRNA involved in the RNAi response is administered to the subject, thereby inhibiting an RNAi response in a cell. In some embodiments, the subject has an abnormal aging disorder such as, for example, Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome, Cockayne's syndrome, ataxia telangiectasia, or Down's syndrome. In some embodiments, inhibiting the RNAi response inhibits aging or extends lifespan.

[0011] In another embodiment, the invention provides heterologous constructs comprising an age-associated gene as described herein or a promoter thereof, and a heterologous sequence such as a regulatory region, a reporter gene, a purification tag, *e.g.*, for production

of a fusion protein, for purification of a gene product, for more efficient expression of the gene or gene product, or for regulated expression of the gene or for expression of a reporter using the gene promoter.

[0012] In one embodiment, methods known to those of skill in the art such as RT-PCR, northern, Southern analysis, cDNA and genomic library cloning, etc. can be used to identify eukaryotic orthologs, *e.g.*, invertebrate, vertebrate, plant, mammalian, and human orthologs, of the age-associated proteins provided herein. In another embodiment, computer sequence analysis can be used to identify orthologs. Such methods optionally include the step of assessing an age associated parameter in a cell in which the suspected ortholog is perturbed.

[0013] In one embodiment, endogenous or recombinant gene products of the age associated genes described herein are purified using the methods described herein, to at least about 50% purity, preferably 60%, 70%, 80%, 90% or higher purity. In another embodiment, the present invention provides a reaction mixture comprising an age-associated protein and another component such as a test compound, an antibody, a peptide, etc.

[0014] In another aspect, the invention features a nucleic acid that includes a regulatory sequence (*e.g.*, a transcriptional regulatory sequence) of an age associated gene or a homolog or ortholog thereof (*e.g.*, a human or other mammalian homolog, as listed below) operably linked to a sequence encoding a detectable protein other than the protein encoded by the gene, *e.g.*, a reporter protein, *e.g.*, a protein that has an epitope tag, that can fluorescence, or that can catalyze a reaction. The invention also provides a transgenic organism that includes at least one cell that includes such a heterologous nucleic acid, and also organisms in which that cell that includes the heterologous nucleic acid also includes at least a second heterologous nucleic acid, *e.g.*, a second reporter gene. The second heterologous nucleic acid can also include a regulatory sequence (*e.g.*, a transcriptional regulatory sequence) of a an age associated gene or a homolog or ortholog thereof (*e.g.*, a human or other mammalian homolog) operably linked to a sequence encoding a detectable protein other than the protein encoded by the gene and other than the protein encoded by the first heterologous nucleic acid. In one embodiment, the two heterologous nucleic acids include regulatory sequences from different classes of genes (*e.g.*, so that one heterologous nucleic acid includes a regulatory sequence from one class, and the other heterologous nucleic acid includes a regulatory sequence from another class). In another embodiment, they include regulatory sequences from the same class of genes. The regulatory sequence can be at least 100, 200, 500 bp, or at

least 1, or 2 kb in length, *e.g.*, between 0.1 and 5 kb, or 0.2 and 3 kb in length. The regulatory sequence can include at least one, two, three or four copies of the GTAAAt/cA motif and/or at least one, two, three or four copies of the CTTATCA motif. In one embodiment, the regulatory sequence includes a region from between about 10 bp 5' of the ATG or first codon to about 400, 500, 700, or 900 bp 5' of the ATG or first codon, or between about 100 bp 5' of the ATG or first codon to about 1, 2, or 5 kb 5' of the ATG or first codon. In one embodiment, the transgenic organism is an invertebrate, *e.g.*, a nematode, *e.g.*, *C. elegans*. For example, the *C. elegans* can include a mutation in one or more of daf-2, daf-16, daf-18, age-1, sir-2 or glp-1 or can be treated with an RNAi specific to such genes.

[0015] Some exemplary human orthologs and homologs of the lifespan genes described herein are listed as follows:

gcy-6	gi|4505435|ref|NP_000897.1| natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A); Natriuretic peptide receptor A/guanylate cyclase A [Homo sapiens] gi|113912|sp|P16066|ANPA_HUMAN Atrial natriuretic peptide receptor A precursor (ANP-A) (ANPRA) (GC-A) (Guanylate cyclase) (NPR-A) (Atrial natriuretic peptide A-type receptor) gi|68381|pir||OYHUAR natriuretic peptide receptor A precursor - human gi|28230|emb|CAA33417.1| ANP-A receptor preprotein (AA -32 to 1029) [Homo sapiens] gi|3297986|dbj|BAA31199.1| natriuretic peptide A type receptor [Homo sapiens] gi|6013455|gb|AAF01340.1|AF190631_1 natriuretic peptide receptor A [Homo sapiens]
gcy-18	gi|14349136|emb|CAC41350.1| guanylate cyclase [Mus musculus] >gi|728861|sp|P18293|ANPA_MOUSE Atrial natriuretic peptide receptor A precursor (ANP-A) (ANPRA) (GC-A) (Guanylate cyclase) (NPR-A) (Atrial natriuretic peptide A-type receptor) gi|2118323|pir||I57963 natriuretic peptide receptor A - mouse gi|473634|gb|AAA66945.1| natriuretic peptide receptor A
C54G4.6 also dod-18	gi|4757794|ref|NP_004183.1| acetylserotonin O-methyltransferase-like; acetylserotonin N-methyltransferase-like [Homo sapiens] gi|3808148|emb|CAA75675.1| ASMTL [Homo sapiens]
C46F4.2 dod-9	gi|12669909|ref|NP_075266.1| long-chain fatty-acid-Coenzymegi|4758332|ref|NP_004449.1| long-chain fatty-acid-Coenzyme gi|19911070|dbj|BAB86900.1| Acyl-CoA synthetase 4 [Homo sap... gi|2960069|emb|CAA73314.1| acyl-CoA synthetase-like protein gi|27469830|gb|AAH41692.1| fatty-acid-Coenzyme A ligase, lo gi|4758330|ref|NP_004448.1| long-chain fatty-acid-Coenzyme gi|7706449|ref|NP_057318.1| fatty-acid-Coenzyme A ligase
F32A5.5 (MIP family)	gi|22538420|ref|NP_536354.2| aquaporin 10; small intestine ... gi|10280624|ref|NP_066190.1| aquaporin 9 [Homo sapiens] gi|4826645|ref|NP_004916.1| aquaporin 3 [Homo sapiens]

	gi|1362754|pir||A57119 aquaporin 3 - human gi|4502187|ref|NP_001161.1| aquaporin 7; aquaporin adipose gi|20137410|sp|Q96PS8|AQPA_HUMAN Aquaporin 10 (Small intest... gi|21912983|dbj|BAC05693.1| aquaporin adipose [Homo sapiens gi|17384411|emb|CAD13298.1| bA251O17.3 (similar to aquapori gi|25815123|emb|CAD38526.1| aquaporin-3 [Homo sapiens gi|18490903|gb|AAH22486.1| aquaporin 1 (channel-forming int... gi|2119185|pir||I52366 uterine water channel - human gi|4502177|ref|NP_000376.1| aquaporin 1; aquaporin 1 (chann... gi|19387211|gb|AAL87136.1| aquaporin 1 [Homo sapiens]

1: NP_000404
hydroxysteroid (17-beta) dehydrogenase 1; Estradiol 17-beta-dehydrogenase-1 [Homo sapiens]
gi|4504501|ref|NP_000404.1|[4504501]

2: Q92506
Estradiol 17 beta-dehydrogenase 8 (17-beta-HSD 8) (17-beta-hydroxysteroid dehydrogenase 8) (Ke6 protein) (Ke-6)
gi|12643402|sp|Q92506|DHB8_HUMAN[12643402]

3: P56937
Estradiol 17 beta-dehydrogenase 7 (17-beta-HSD 7) (17-beta-hydroxysteroid dehydrogenase 7)
gi|8134404|sp|P56937|DHB7_HUMAN[8134404]

4: P51659
Estradiol 17 beta-dehydrogenase 4 (17-beta-HSD 4) (17-beta-hydroxysteroid dehydrogenase 4)
gi|1706396|sp|P51659|DHB4_HUMAN[1706396]

5: P37059
Estradiol 17 beta-dehydrogenase 2 (17-beta-HSD 2) (Microsomal 17-beta-hydroxysteroid dehydrogenase) (20 alpha-hydroxysteroid dehydrogenase) (20-alpha-HSD) (E2DH)
gi|544152|sp|P37059|DHB2_HUMAN[544152]

6: P14061
Estradiol 17 beta-dehydrogenase 1 (17-beta-HSD 1) (Placental 17-beta-hydroxysteroid dehydrogenase) (20 alpha-hydroxysteroid dehydrogenase) (20-alpha-HSD) (E2DH)
gi|118554|sp|P14061|DHB1_HUMAN[118554]

7: S59136
estradiol 17beta-dehydrogenase (EC 1.1.1.62) type 4 - human
gi|2134658|pir||S59136[2134658]

8: S43928
estradiol 17beta-dehydrogenase (EC 1.1.1.62) type 3 - human

gi|1085271|pir||S43928[1085271]

9: A47287
estradiol 17beta-dehydrogenase (EC 1.1.1.62) type 2 - human
gi|539530|pir||A47287[539530]

10: DEHUE7
estradiol 17beta-dehydrogenase (EC 1.1.1.62) type 1 [validated] - human
gi|65913|pir||DEHUE7[65913]

11: NP_803882
cytochrome P450, family 20, subfamily A, polypeptide 1 isoform 1; cytochrome P450 monooxygenase [Homo sapiens]
gi|29171730|ref|NP_803882.1|[29171730]

12: NP_065725
cytochrome P450, family 20, subfamily A, polypeptide 1 isoform 2; cytochrome P450 monooxygenase [Homo sapiens]
gi|29171727|ref|NP_065725.2|[29171727]

13: NP_000932
P450 (cytochrome) oxidoreductase; Cytochrome P-450 reductase [Homo sapiens]
gi|24307877|ref|NP_000932.1|[24307877]

14: NP_000766
cytochrome P450, family 2, subfamily J, polypeptide 2; cytochrome P450, subfamily IIJ (arachidonic acid epoxygenase) polypeptide 2; microsomal monooxygenase; flavoprotein-linked monooxygenase [Homo sapiens]
gi|18491008|ref|NP_000766.2|[18491008]

15: NP_476437
cytochrome P450, family 3, subfamily A, polypeptide 43 isoform 3; cytochrome P450 polypeptide 43; cytochrome P450, subfamily IIIA, polypeptide 43 [Homo sapiens]
gi|16933535|ref|NP_476437.1|[16933535]

16: NP_476436
cytochrome P450, family 3, subfamily A, polypeptide 43 isoform 2; cytochrome P450 polypeptide 43; cytochrome P450, subfamily IIIA, polypeptide 43 [Homo sapiens]
gi|16933533|ref|NP_476436.1|[16933533]

17: NP_000774
cytochrome P450, family 26, subfamily A, polypeptide 1 isoform 1; cytochrome P450, subfamily XXVIA, polypeptide 1; P450, retinoic acid-inactivating, 1; retinoic acid-metabolizing cytochrome; retinoic acid 4-hydroxylase [Homo sapiens]
gi|16933530|ref|NP_000774.2|[16933530]

18: NP_476498

cytochrome P450, family 26, subfamily A, polypeptide 1 isoform 2; cytochrome P450, subfamily XXVIA, polypeptide 1; P450, retinoic acid-inactivating, 1; retinoic acid-metabolizing cytochrome; retinoic acid 4-hydroxylase [Homo sapiens]
gi|16933528|ref|NP_476498.1|[16933528]

19: NP_061950
UDP glycosyltransferase 1 family, polypeptide A7; UDP-glucuronosyltransferase 1A7 [Homo sapiens]
gi|29789084|ref|NP_061950.1|[29789084]

20: NP_061948
UDP glycosyltransferase 1 family, polypeptide A10; UDP-glucuronosyltransferase 1A10 [Homo sapiens]
gi|29789078|ref|NP_061948.1|[29789078]

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1 illustrates data showing that daf-2 and daf-16 bacterial RNAi lower mRNA levels. (A) RT-PCR analysis of daf-2 mRNA following RNAi treatment. Shown are RT-PCR products from serial dilutions of total RNA isolated from control animals grown on bacteria containing vector only (lanes 1-5) or on bacteria expressing daf-2 dsRNA (lanes 6-10). (a), RNAi was initiated at hatching, and RNA was harvested at L4. (b), RNAi was initiated on day 8 of adulthood and harvested on day 10 of adulthood. (B) RT-PCR analysis of daf-16 mRNA following RNAi treatment. Conditions were the same as in (A), except animals were treated with bacteria expressing daf-16 dsRNA.

[0017] Figure 2 illustrates data showing that daf-2 RNAi affects lifespan and reproduction at different times. The time at which animals were transferred onto bacteria expressing daf-2 dsRNA is shown in the upper right corner of each panel. Blue lines represent the lifespans of animals grown on control bacteria carrying the RNAi vector alone, and red lines represent lifespans of animals grown on bacteria expressing daf-2 dsRNA. Reproductive profiles of animals in the concurrent lifespan assays are depicted to the right of each lifespan profile. The percent of total progeny produced at each 12-hour time interval is shown. Scales are identical for all graphs. Reproductive profiles of animals treated with bacteria expressing daf-2 dsRNA after day 4 of adulthood are not shown since these animals were post-reproductive. For statistics, see Table 1.

[0018] Figure 3 illustrates data showing that daf-16 RNAi affects the lifespan and reproduction of daf-2(e1370) mutants at different times during the life cycle. The time that daf-2(e1370) animals were transferred onto daf-16 RNAi bacteria is shown in the upper right

corner of each panel. Blue lines, lifespans of daf-2(e1370) animals grown on bacteria carrying the RNAi vector alone; red lines, lifespans of daf-2(e1370) animals grown on daf-16 RNAi bacteria. Note, scales of graphs A-N are identical and O-S are identical. Insets: T0 is set to the day at which RNAi treatment was initiated in the experimental population. For statistics, see Table 1. Reproductive profiles of animals in the concurrent lifespan assays are depicted to the right of each lifespan profile. The percent of total progeny produced at each 12-hour time interval is shown. Blue, daf-2(e1370) mutant animals grown on control bacteria; red, daf-2(e1370) mutant animals grown on bacteria expressing daf-16 dsRNA. Reproductive profiles of animals cultured on daf-16 RNAi bacteria after day 5 of adulthood are not shown since the animals were post-reproductive.

[0019] Figure 4 illustrates data showing that loss of daf-2 function during development does not increase lifespan. Wild-type animals were grown on bacteria expressing daf-2 dsRNA from hatching until the first day of adulthood and then transferred to bacteria expressing dsRNA of dcr-1. Red line, lifespans of wild-type animals grown on daf-2 RNAi bacteria during development and then shifted during day 1 of adulthood to bacteria expressing dcr-1 RNAi bacteria. Blue line, lifespan of wild-type animals grown on the control RNAi bacteria during development and then shifted during day 1 of adulthood to dcr-1 RNAi bacteria. Black line, lifespan of wild-type animals grown of daf-2 RNAi bacteria during development and adulthood. Lifespans were conducted at 25oC. For statistics, see Table 1.

[0020] Figure 5 illustrates data showing conditional expression of daf-2 using daf-2 RNAi and dicer RNAi.

[0021] Figure 6 illustrates data showing conditional expression of an electron transport chain (ETC) component using cco-1 RNAi and dicer RNAi.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

[0022] Double-stranded RNA-mediated interference (RNAi) provides a sequence specific mechanism for inhibiting gene expression (*see, e.g.*, Fire *et al., Nature* 391:806-811 (1998) McManus *et al., J. Immunol.* 169(10):5754-60 (2002), McManus and Sharp, *Nature Rev. Genet.* 3:737 (2002), Elbashir, *et al.*, Genes Dev. 15:188 (2001) and McManus *et al., RNA* 8(6):842-50 (2002), and WO 99/32619)). As explained above, the RNAi response is

extremely stable and can be heritable. Therefore, an important factor for use of RNAi technology as a therapeutic is the ability to turn off the RNAi response once it has been established, or the ability to conditionally turn on and off an RNAi response.

[0023] The present invention provides methods of regulating the RNAi response and is based on the discovery that inhibition of proteins involved in the RNAi response (*e.g.*, dcr-1, *C. elegans*: NM_075691, NM_075876, NM_070776, and NM_072209; human: NM_012154, NM_030621, and NM_177438) can conveniently be used to inhibit the response. Other C. elegans genes and their human homologs that can be used to regulate RNAi include rde-1 (NM171525); smg-5 (NM_059528), ego-1 (NM_059731), and red-4 (NM_066864). According to the methods of the invention, a dsRNA involved in the RNAi response is contacted with a cell, thereby inhibiting the RNAi response. In an exemplary embodiment, inhibition of the RNAi response inhibits aging or increases lifespan. Inhibition of the RNAi response can conveniently be detected by detecting expression of aging associated proteins using the methods described herein.

[0024] DEFINITIONS

[0025] The terms "DICER" or "dcr-1" refers to the RNAse III as described in, *e.g.*, Knight and Bass, *Science* 2269-71 (2001) or a homolog or ortholog thereof, preferably a human ortholog. *C. elegans* dcr-1 nucleotide and protein sequences are accession nos. NM_075691, NM_075876, NM_070776, and NM_072209. Human dcr-1 nucleotide and protein sequences are accession nos. NM_012154, NM_030621, and NM_177438. Dicer genes of the invention and other genes of the invention such as rde-1, smg-5, ego-1 and rde-4, hybridize under stringent conditions to the nucleotide sequences listed as accession numbers herein, or have at least about 80% identity to the amino acid or nucleotide sequences listed as accession numbers herein, preferably at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity.

[0026] A "target gene" refers to any gene suitable for regulation of expression using RNAi, including both endogenous chromosomal genes and transgenes, as well as episomal or extrachromosomal genes, mitochondrial genes, chloroplastic genes, vir al genes, bacterial genes, animal genes, plant genes, protozoal genes and fungal genes. In a preferred embodiment, the target gene is a gene involved in the RNAi process, *e.g.*, dcr-1. Additional preferred target genes include lifespan genes, such as those listed in Murphy *et al., Nature* 424:277-283 (2003), herein incorporated by reference in its entirety.

[0027] An "siRNA" or "RNAi" refers to a nucleic acid that forms a double stranded RNA ("dsRNA"), which double stranded RNA has the ability to reduce or inhibit expression of a gene or target gene when the siRNA expressed in the same cell as the gene or target gene. The term "RNAi" has also been used to refer to the process of inhibition. "siRNA" thus refers to a double stranded RNA formed by complementary strands. The complementary portions of the siRNA that hybridize to form the double stranded molecule typically have substantial or complete identity. In one embodiment, an siRNA refers to a nucleic acid that has substantial or complete identity to a target gene, *e.g.*, dcr-1, and forms a double stranded siRNA. The sequence of the siRNA can correspond to the full length target gene, or a subsequence thereof. Typically, the siRNA is at least about 15-50 nucleotides in length (*e.g.*, each complementary sequence of the double stranded siRNA is 15-50 nucleotides in length, and the double stranded siRNA is about 15-50 base pairs in length, preferable about preferably about 20-30 base nucleotides, preferably about 20-25 nucleotides in length, *e.g.*, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length, most preferably 21-22 base pairs in length.

[0028] "Inverted repeat" refers to a nucleic acid sequence comprising a sense and an antisense element positioned so that they are able to form a double stranded siRNA when the repeat is transcribed. The inverted repeat may optionally include a linker or a heterologous sequence between the two elements of the repeat. The elements of the inverted repeat have a length sufficient to form a double stranded RNA. Typically, each element of the inverted repeat is about 15 to about 100 nucleotides in length, preferably about 20-30 base nucleotides, preferably about 20-25 nucleotides in length, *e.g.*, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[0029] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0030] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*,

"Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0031] Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36C is typical for low stringency amplification, although annealing temperatures may vary between about 32C and 48C depending on primer length. For high stringency PCR amplification, a temperature of about 62C is typical, although high stringency annealing temperatures can range from about 50C to about 65C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90C - 95C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

[0032] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, *e.g.*, and Current Protocols in Molecular Biology, ed. Ausubel, *et al.*

[0033] The phrase "inhibiting expression of a target gene" refers to the ability of a siRNA or dsRNA of the invention to initiate gene silencing of the target gene. To examine the extent of gene silencing, samples or assays of the organism of interest or cells in culture expressing a particular construct are compared to control samples lacking expression of the construct. Control samples (lacking construct expression) are assigned a relative value of 100%. Inhibition of expression of a target gene is achieved when the test value relative to the control is about 90%, preferably 50%, more preferably 25-0%. Suitable assays include those described below in the Example section, *e.g.*, examination of protein or mRNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0034] Orthologs are biopolymeric sequences (*e.g.*, nucleic acid or polypeptide sequences) that are found in different species, yet have sequence similarity (*e.g.*, at least 20% sequence identify within a functional protein domain), and perform similar functions. In one embodiment (*e.g.*, wherein multiple homologs are present) an ortholog is most homology to a reference sequence relative to other available sequences. In one embodiment, orthologs can be assigned by comparing numerous sequences to identify the best match-up. See, *e.g.*, *Science* 1997 Oct 24;278(5338):631-7 and *Nucleic Acids Res* 2001 Jan 1; 29(1):22-28 for some exemplary methods and resource for assigning orthologs based on complete genome coverage.

[0035] The phrase "modulate expression" or "modulates expression" refers to increasing or decreasing expression of aging associated genes and proteins includes the determination of a parameter that is indirectly (*e.g.*, upstream or downstream biochemical or genetic effects) or directly under the influence of aging associated proteins, *e.g.*, a chemical or phenotypic effect, such as the ability to increase or decrease lifespan (*see, e.g.*, Kenyon *et al., Nature* 366:461-464 (1993); Hsin & Kenyon, *Nature* 399:362-366 (1999); Apfeld & Kenyon, *Cell* 95:199-210 (1998); and Lin *et al., Nature Genet.* 28:139-145 (2001)) or, *e.g.*, a physical effect such as ligand, cofactor or substrate binding or inhibition of ligand, cofactor or substrate binding. A functional effect therefore includes ligand, cofactor and substrate binding activity; changes in gene expression and gene expression levels in cells; changes in post transcriptional modification of a protein, *e.g.*, phosphorylation or glycosylation; reporter gene or marker expression; changes in abundance and cellular localization; enzymatic activity; cellular half life; redox state; and structural conformation, etc.; and age-associated

parameters, *i.e.*, characteristics of young or old cells or organisms such as stress resistance, lifespan, doubling time, telomere length, physiological characteristics, appearance, disease states, etc. "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities. The functional effect can be measured in a host cell, organelle (*e.g.*, isolated mitochondria), host cell membrane, isolated organelle membrane (*e.g.*, isolated mitochondrial membrane), cellular extract, organelle extract (*e.g.*, mitochondrial extract) or host organism.

[0036] By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of aging associated proteins or genes, *e.g.*, measuring physical and chemical or phenotypic effects. Such functional effects can be measured by any means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index); hydrodynamic (*e.g.*, shape); chromatographic; or solubility properties for the protein; measuring inducible markers or transcriptional activation of the protein; measuring binding activity or binding assays, *e.g.* binding to antibodies; measuring changes in ligand binding activity; measuring cellular proliferation or lifespan; measuring cell surface marker expression; measurement of changes in protein levels for associated sequences; measurement of RNA stability; phosphorylation or dephosphorylation; signal transduction, *e.g.*, receptor-ligand interactions, second messenger concentrations (*e.g.*, cAMP, IP3, or intracellular Ca^{2+}); identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), *e.g.*, via chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays.

[0037] "Ligand" refers to a molecule that is specifically bound by a protein.

[0038] "Substrate" refers to a molecule that binds to an enzyme and is part of a specific chemical reaction catalyzed by the enzyme.

[0039] "Cofactor" refers to an additional component required for activity of an enzyme. (Leninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). A cofactor may be inorganic such as Fe, Cu, K, Ni, Mo, Se, Zn, Mn or Mg ions, or an organic molecule also known as a coenzyme. Coenzymes include flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), heme, coenzyme A, pyrodoxal phosphate, thiamine pyrophosphate, 5'-deoxyadenosylcobalamine, biocytin, tetrahydrofolate, retinal, and 1,25-dihydroxycholecalciferol. A co-factor can also include a protein subunit bound to the co-factor.

[0040] "Inhibitors", "activators", and "modulators" of aging associated genes and proteins are used to refer to activating, inhibitory, or modulating molecules identified using *in vitro* and *in vivo* assays of aging associated proteins and genes. Inhibitors are compounds that, *e.g.*, bind to, partially or totally block activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity or expression of aging associated proteins and genes, *e.g.*, antagonists. "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate aging associated proteins. Inhibitors, activators, or modulators also include genetically modified versions of aging associated proteins and genes, *e.g.*, versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, antibodies, antisense molecules, ribozymes, small chemical molecules and the like. Such assays for inhibitors and activators include, *e.g.*, expressing aging associated proteins *in vitro*, in cells, or cell membranes, applying putative modulator compounds, and then determining the functional effects on activity, as described above.

[0041] "Biological sample" include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood, sputum, tissue, cultured cells, *e.g.*, primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, *e.g.*, *C. elegans*, most preferably a mammal such as a primate *e.g.*, chimpanzee or human; cow; dog; cat; a rodent, *e.g.*, guinea pig, rat, mouse; or a rabbit.

[0042] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0043] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifica lly, degenerate codon substitutions may be achieved by generating sequences in which the third

position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0044] A particular nucleic acid sequence also implicitly encompasses "splice variants." Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. "Splice variants," as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition.

[0045] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, about 50% identity, preferably 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (*e.g.*, the *C. elegans* proteins provided herein), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

[0046] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if

necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0047] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0048] A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to

calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0049] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. In one embodiment, a "protein" or "polypeptide" includes a plurality of subunit chains, *e.g.*, the quaternary structure of the protein or polypeptide is multimeric (*e.g.*, homo- or hetero-dimeric). Accordingly, a "protein" or "polypeptide"may be a complex of different subunit chains. In another embodiment, a "protein" or "polypeptide" refers to a single chain.

[0050] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0051] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0052] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

[0053] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs and orthologs, and alleles of the invention.

[0054] The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I),

Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g.*, Creighton, *Proteins* (1984)).

[0055] Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, *see, e.g.*, Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains, *e.g.*, extracellular domains, transmembrane domains, and cytoplasmic domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 15 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β-sheet and α-helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

[0056] A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

[0057] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[0058] The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the

same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, *e.g.*, a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (*e.g.*, a fusion protein).

[0059] "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

[0060] An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0061] Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single

chain Fv) or those identified using phage display libraries (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990))

[0062] For preparation of antibodies, *e.g.*, recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

[0063] A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[0064] In one embodiment, the antibody is conjugated to an "effector" moiety. The effector moiety can be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the antibody modulates the activity of the protein.

[0065] The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein.

For example, polyclonal antibodies raised to aging associated proteins, polymorphic variants, alleles, orthologs, and conservatively modified variants, or splice variants, or portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with aging associated proteins and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

CONDITIONAL GENE EXPRESSION

[0066] According to the methods of the present invention, dsRNA of genes involved in the RNAi response (*e.g.*, dcr-1) can be delivered to cells or to an organism to inhibit the response. Once dsRNA is delivered to cells or to an organism, endogenous components of the cell or organism can trigger RNA interference (RNAi) which silences expression of genes that include the target sequence involved in RNA1 (*e.g.*, dcr-1). Silencing of gene expression can be detected by direct detection of the gene product involved in the RNAi response or by detection of gene products from genes modulated by the gene involved in the RNAi response (*e.g.*, aging associated genes). By using dsRNA to inhibit expression of genes involved in RNAi, such as dcr-1, RNAi responses can be conditionally regulated. For example, expression target genes or sequences involved in lifespan can be conditionally regulated by silencing the lifespan genes using RNAi, and then using dsRNA of an RNAi component to turn off the RNAi machinery. Preferred lifespan genes are listed in Murphy *et al., Nature* 424:277-283 (2003), herein incorporated by reference in its entirety.

[0067] In an exemplary embodiment, vectors encoding dsRNA involved in the RNAi response are transfected into suitable host cells (*e.g.*, bacteria) and the host cells are administered to a subject (*e.g.*, a nematode).

[0068] dsRNA can be produced using any means known in the art. DNA molecules for transcribing dsRNA are disclosed in, *e.g.*, U.S. Patent No. 6,573,099, and in U.S. Patent Application Publication Nos. 2002/0160393 and 2003/0027783. For example, dsRNA can be produced by transcribing a cassette in both directions, for example, by including a T7 promoter on either side of the cassette. dsRNA can be produced by transcribing a cassette in

both directions, for example, by including a T7 promoter on either side of the cassette. The insert in the cassette is selected so that it includes a sequence complementary to a gene to be attenuated, *i.e.*, a gene encoding a protein involved in the RNAi response (*e.g.*, dcr-1 or ins-7). The sequence need not be full length, for example, an exon, or at least 50 nucleotides, preferably from the 5' half of the transcript, *e.g.*, within 300 nucleotides of the ATG. See also, the HiScribe™ RNAi Transcription Kit (New England Biolabs, MA) and Fire, (1999) *Trends Genet.* 15, 358–363. dsRNA can be digested into smaller fragments. See, *e.g.*, US Patent Application 2002-0086356 and 2003-0084471. In one embodiment, an siRNA is used. DNA molecules for transcribing siRNA are reviewed in Tuschl and Borkhardt, Molecular Interventions, 2:158 (2002). siRNAs are small double stranded RNAs (dsRNAs) that optionally include overhangs. For example, the duplex region is about 18 to 25 nucleotides in length, *e.g.*, about 19, 20, 21, 22, 23, or 24 nucleotides in length. Typically the siRNA sequences are exactly complementary to the target mRNA. dsRNAs and siRNAs in particular can be used to silence gene expression in mammalian cells (*e.g.*, human cells). See, *e.g.*, Clemens, *et al.* (2000) *Proc. Natl. Sci. USA* 97, 6499–6503; Billy, *et al.* (2001) *Proc. Natl. Sci. USA* 98, 14428–14433; Elbashir *et al.* (2001) *Nature* 411(6836):494-8; Yang, *et al.* (2002) *Proc. Natl. Acad. Sci. USA* 99, 9942–9947.

GENES AND GENE PRODUCTS THAT REGULATE AGING

[0069] As explained above, detection of genes that regulate the aging process (or their products) after administration of dsRNA involved in the RNAi response can be used to monitor the inhibition of the RNAi response. Genes that modulate the aging process include, for example, cellular stress-response genes, antimicrobial genes, metabolic genes, steroid or lipid-soluble hormone synthesis genes, and fatty acid desaturation genes. Exemplary genes that modulate the aging process include, but are not limited to genes that encode cytochrome P450, estradiol-17-β-dehydrogenases, alcohol/short-chain dehydrogenases, esterases, UDP-glucuronosyltransferases, aminopeptidases, carboxypeptidases, amino-oxidases, aminoacylases, oligopeptide transporters, metallothioneins, receptor guanylate cyclases, mitochondrial superoxide dismutases, catalases, lysosymes, saposins, vitellogenins, glutathione-S-transferases, heat-shock proteins, heat shock factors, F-box/cullin/Skp proteins, isocitrate lyases, malate synthases ASMTL, insulins, and insulin growth factors. Additional genes, and gene products that modulate the aging process can be identified using any method known in the art, *e.g.*, RNAi analysis; microarray analysis; chemical mutagenesis; mammalian complementation assays for age-associated proteins; yeast two hybrid assays,

immunoprecipitation; alteration in age-associated reporter gene expression or localization (*e.g.*, daf-2 or daf-16); overexpression, underexpression, or knockout of gene expression, etc. Suitable controls include organisms with altered lifespan, *e.g.*, by mutation or RNAi. These assays can be used with eukaryotic organisms, cells, and organelles such as mitochondria. The genes and gene products associated with a mutation are then identified and used to analyze the aging process at a molecular level. Genes and gene products that regulate the aging process can be identified under normal aging conditions. Patterns of gene expression that correlate with normal or abnormal aging can also be used to identify genes associated with aging. The aging process has likely been conserved throughout evolution. Thus, genes and gene products that regulate the aging process in one species will be useful to identify similar or orthologous genes and gene products in divergent species.

A. Manifestations of the Aging Process

[0070] The most obvious disruption of the aging process is a change in lifespan of an individual. Lifespan can either be increased or decreased by a mutation in a gene that participates in the aging process or, as shown here, by another intervention, *e.g.*, RNAi mediated silencing of such a gene. In addition, for all eukaryotic organisms other physical characteristics can be used to distinguish young individuals from older individuals. Thus, at an organismal level, a mutation that affects the aging process will usually affect the lifespan of an individual and may also affect other aging characteristics of that individual. Such manifestations of the aging process are known as "age-associated parameters," *e.g.*, indicia from Nomarski analysis, stress resistance, appearance, physiological changes, disease states, loss of doubling capacity, changes in differentiated phenotype, indirect effects such as fusion protein expression and localization or posttranscriptional modification, etc., are described in more detail below.

[0071] Those of skill in the art will recognize that the aging process can also be manifested at an organismal level or at a cellular level. While a list of characteristics of aging is provided below, it is not exhaustive and other characteristics of the aging process may also be analyzed within the scope of the present invention.

[0072] Characteristics of aging can be distinguished at the organismal level and may be species specific. For example, characteristics of older human individuals include skin wrinkling, graying of the hair, baldness, cataracts, hypermelanosis, osteoporosis, cerebral

cortical atrophy, lymphoid depletion, thymic atrophy, increased incidence of diabetes type II, atherosclerosis, cancer, and heart disease (Nehlin *et al.*, *Annals NY Acad. Sci.*, 980:176-179 (2000)). Other characteristics of mammalian aging include the following: weight loss; lordokyphosis (hunchback spine); absence of vigor; lymphoid atrophy; decreases in bone density, dermal thickness, and subcutaneous adipose tissue; decreased ability to tolerate stresses, such as wound healing, anesthesia, and response to hematopoietic precursor cell ablation; sparse hair; liver pathologies; atrophy of intestinal villi; skin ulceration; amyloid deposits; and joint diseases (Tyner *et al.*, *Nature* 415:45-53 (2002)).

[0073] Many diseases and disorders also are associated with aging or increased age. Exemplary age-related diseases and disorders include: cancer (*e.g.*, breast cancer, colorectal cancer, CCL, CML, prostate cancer); skeletal muscle atrophy; adult-onset diabetes; diabetic nephropathy, neuropathy (*e.g.*, sensory neuropathy, autonomic neuropathy, motor neuropathy, retinopathy); obesity; bone resorption; age-related macular degeneration, ALS, , Bell's Palsy, atherosclerosis, cardiac diseases (*e.g.*, cardiac dysrhythmias, chronic congestive heart failure, ischemic stroke, coronary artery disease and cardiomyopathy), chronic renal failure, type 2 diabetes, ulceration, cataract, presbiopia, glomerulonephritis, Guillan-Barre syndrome, hemorrhagic stroke, short-term and long-term memory loss, rheumatoid arthritis, inflammatory bowel disease, neurodegenerative disorders (*e.g.*, Alzheimer's, Huntington's, Parkinson's), multiple sclerosis, SLE, Crohn's disease, osteoarthritis, pneumonia, and urinary incontinence. In addition, many disorders associated with protein aggregation (*e.g.*, polyglutamine aggregation, amyloid formation, etc) or protein misfolding can also be age-related. Symptoms and diagnosis of diseases are well known to medical practitioners. A compound identified by a method described herein can be used to ameliorate at least one symptom of such diseases and disorders. Similarly, one or more genes described herein can be used to evaluate a risk, association, or status of such diseases and disorders.

[0074] Careful observation reveals characteristics of the aging process in other eukaryotes, including invertebrates. For example, characteristics of aging in the model nematode *C. elegans* as observed by Nomarski analysis include slow movement, flaccidity, yolk accumulation, intestinal autofluorescence (lipofuscin), loss of ability to chew and expel (distended oral and anal cavities), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities).

[0075] Characteristics of aging can also be observed in cultured cells and also in mitochondria. Note that many of these characteristics can also be observed in animals. Normal eukaryotic cells have a defined lifespan when taken from the organism grown in culture. These "primary" tissue culture cells are cells that have neither been immortalized nor acquired a transformed phenotype. The primary cells will divide a defined number of times in culture and then die (reviewed in Campisi, *Exper. Geron.* 36:6-7-618 (2001)). Cellular aging is also characterized by changes other than loss of doubling capability, *e.g.* changes in apoptotic death and changes in differentiated phenotypes (*Id.*). In some cases, cellular characteristics of aging can also be observed in immortalized or transformed cell lines. Aging cells also show stress resistance, *e.g.*, free radical generation and H_2O_2 resistance. Age-related bio-markers, gene, and protein expression patterns may also be used to determine or measure aging.

[0076] Finally, aging can be assessed indirectly, by an aging related functional effects (phenotypic, physical, and chemical effects), *e.g.*, gene expression (*e.g.*, transcript abundance), protein abundance/localization/modification state, chromatin structure, signal transduction, second messenger levels, marker expression, phosphorylation, posttranscriptional modification, reporter gene expression, reporter or fusion protein localization, etc. Such effects can often be monitored when examining upstream or downstream genetic or biochemical pathways of an aging associated gene. Such effects can also be monitored using the aging associated gene.

B. Isolation of Genes Associated with Aging

[0077] Those of skill in the art will recognize that aging associated nucleic acids and proteins may be conserved in divergent species. Thus, the sequence of a nucleic acid or protein associated with aging in one species can be used to identify aging associated nucleic acids and proteins from other species, as well as genetic and biochemical pathways for the aging associated genes. For example, using methods described in this specification, aging associated genes identified in *C. elegans* can be used to identify aging associated genes or proteins in humans or other higher eukaryotes.

Isolation of genes and gene products associated with aging using classical genetic methods.

[0078] Using classical genetic methods (random genomic mutagenesis), aging mutants are be generated by mutagenesis. The mutagenesis protocol will depend on the organism. For example, some eukaryotic organisms can be randomly mutagenized chemically by treatment with compounds like ethane methyl sulfonate (EMS) or can be mutagenized by exposure to UV or gamma irradiation. Preferably, these compounds would be used on organisms such as mammalian cells, yeast, *C. elegans*, *Drosophila melanogaster*, or zebrafish.

[0079] Mutants in the aging process will preferably be characterized by an increase or a decrease in lifespan (*e.g.*, at least 10, 20, 40, 50, 70, 90, 100, 120, 150% greater than wild-type, or at least 10, 20, 30, 40, 50, or 60% less than wild-type). Mutants in the aging process will also preferably exhibit a temporal change in expression of an aging characteristic, including those listed above.

[0080] Those of skill in the art will recognize that mutants can be generated in many ways depending on the organism and phenotypes studied. Typically, the mutagenesis process decreases, increases, or changes gene activity. Examples of such mutants include age-1, daf-2, and daf-16 in *C. elegans*.

Isolation of genes and gene products associated with aging using gene inactivation.

[0081] In another embodiment, aging mutants are made by inactivation of a gene of interest, using methods other than classical genetic mutagenesis methods. The gene of interest can be inactivated, *e.g.*, using dsRNA inhibition, by using antisense technology, or can be inactivated by homologous recombination. The inactivation can take place in a multicellular organism or in cultured cells. For example, the p66 gene has been inactivated from mice using homologous recombination, creating a mouse with a longer lifespan than wildtype. Transgenic mice of interest which show lifespan increase include Ames dwarf mutant mice, p66(-/-) knockout mice, alpha MUPA and MGMT transgenic mice (*see, e.g.*, Anisimov, *Mech Aging Dev.* 122:1221-1255 (2001); Lithgow & Andersen, *Bioessays* 22:410-413 (2000)).

[0082] dsRNA inhibition can also be used to screen a large number of genes for a phenotype. DNA fragments corresponding to predicted genes are cloned into a vector

between two bacterial promoters in inverted orientation. The library is then transformed into a bacterial strain capable of expressing the DNA fragments. The transformed bacteria or the library DNA alone is then introduced into the experimental organism. If desired, inducible promoters can be used and expression of the inhibitory dsRNA can be induced during a particular time of development or under desired conditions.

[0083] A preferred embodiment uses a library whose members each include a DNA fragment from *C. elegans*. Each library member is transformed into *E. coli* and the *E. coli* fed to the worms. The DNA fragments are under the control of T7 promoters. The bacteria express a T7 polymerase that is inducible by IPTG, rendering expression of the inhibitory dsRNA inducible by IPTG.

Isolation of genes and gene products associated with aging using overexpression.

[0084] In another embodiment, aging mutants are made by overexpressing a gene associated with aging, using methods other than classical genetic mutagenesis methods.. The gene associated with aging is cloned into a vector under the control of a promoter appropriate for the experimental system. The expression vector is then introduced into the experimental system. The overexpression can take place in either a multicellular organism or in cultured cells.

Isolation of genes and gene products associated with aging using naturally occurring mutants.

[0085] Aging mutants can also occur naturally. Those of skill in the art will recognize that such mutants do exist and can be used in the present invention. For example, in humans, several premature aging syndromes have been characterized including Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome, Cockayne's syndrome, ataxia telangiectasia, and Down's syndrome. Where appropriate, cells from an individual afflicted with an aging syndrome can be studied, rather than the whole organism.

Isolation of genes and gene products associated with aging using genetic or biochemical pathways known to regulate aging.

[0086] Genetic analysis can also be used to delineate regulatory pathways and determine functional relationships between genes and gene products. In the case of a complex biological process such as aging, more than one regulatory pathway may regulate the aging

process. Those of skill in the art will recognize that genetic analysis of mutants can be used to characterize regulatory pathways and determine relationships between genes. Of course, it also possible to use RNA interference to modulate gene activity in analyzing the regulatory pathways and relationships.

[0087] An example of genetic analysis of a regulatory pathway is found in *C. elegans*. The *daf-2* gene encodes an insulin/IGF-1 receptor homolog. Mutations that lower the level of *daf-2* result in animals that have enhanced lifespans. (*For review see* Guarente and Kenyon, *Nature* 408:255-262 (2000)). *daf-16* encodes a forkhead transcription factor homolog that acts downstream of *daf-2* and is required for *daf-2* activity. *daf-16* mutants have short lifespans. Newly isolated mutations can be analyzed for interaction with the *daf2/daf16* pathway. In that way, genes and gene products can be assigned to a regulatory pathway.

[0088] In addition, genes that interact with the pathway can be identified by using an appropriate mutant screen. For example, the *C. elegans* protein DAF-16 is a transcriptional activator. A fusion protein between DAF-16 and green fluorescent protein (DAF-16/GFP) can be used to identify the cellular location of the protein. In wild-type animals the protein is localized throughout cells. In long-lived *daf-2* mutants, DAF-16 is localized to the nucleus.

[0089] Those of skill in the art will recognize that the localization of DAF-16/GFP can be used to identify mutants that perturb the *daf2/daf16* pathway. Localization of DAF-16/GFP to the nucleus can be used to screen for drugs that enhance lifespan or mutations that enhance lifespan. A similar fusion using an end product of the pathway, superoxide dismutase (SOD-3), can be similarly used. Levels of fluorescence from SOD-3/GFP can be followed by microscopy. Those of skill in the art will recognize that expression of SOD-3/GFP can be used to screen for long-lived mutants.

Isolation of genes and gene products associated with aging using changes in expression levels.

[0090] Those of skill in the art will recognize that levels of messenger RNA can be measured during the aging process. For aging associated proteins, changes in mRNA levels can be detected either during normal aging process or when comparing an aging mutant to a wild-type individual. Changes in mRNA levels can be measured using techniques known to those of skill in the art, including microarrays, northern blots, and RT PCR.

[0091] Aging associated genes can be identified through the use of microarrays where changes in expression of mRNA levels under different conditions or at different times of

development can be assayed. mRNA levels can also be analyzed in aging mutants to identify genes that are affected by increases or decreases in lifespan.

[0092] Microarrays are made by methods known to those of skill in the art, or are purchased. Gene expression profiles for the genes described herein can be generated and used for comparison to identify other age-associated genes. The profile can be generated using a microarray, or by other means. The profiles can be derived from animals, cells, mitochondria, or other suitable sources expressing the genes of interest, *e.g.*, RNAi treated cells or animals. Such profiles can be stored as computer files and analyzed or compared to identify additional genes using algorithms known to those of skill in the art.

[0093] Moreover, a gene identified by any method, *e.g.*, transcript or protein profiling, RNAi, or genetic mutation, can then by analyzed by one of the other methods. For example, the activity of a gene whose transcription is correlated with aging can altered using RNAi. Further, chromosomal deficiencies and genetic mutations can be identified in the gene of interest. These exemplary alterations can be used to evaluate the contribution of a gene or gene product to the aging phenotype. The functional relevance of genes so identified can be tested with mutants or RNAi.

ISOLATION OF NUCLEIC ACIDS ENCODING AGING ASSOCIATED PROTEINS

[0094] This invention can include use of routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

[0095] Aging associated protein-encoding nucleic acids, polymorphic variants, orthologs, and alleles can be isolated using the *C. elegans* genes provided herein using, *e.g.*, moderate or low stringent hybridization conditions, by screening libraries, by analyzing a sequence database, and/or by synthetic gene construction. Alternatively, expression libraries can be used to clone aging associated proteins, polymorphic variants, orthologs, and alleles by detecting expressed homologs immunologically with antisera or purified antibodies made against *C. elegans* or mammalian aging associated proteins or portions thereof or by complementation, *e.g.*, of a *C. elegans* phenotype. In a preferred embodiment, human nucleic acid libraries are screened for homologs of *C. elegans* genes or proteins that are associated with aging.

[0096] To make a cDNA library, one can choose a source that is rich in the RNA of choice. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfixed into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.,* Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*).

[0097] For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al., Proc. Natl. Acad. Sci. USA.,* 72:3961-3965 (1975).

[0098] An alternative method of isolating aging associated protein-encoding nucleic acid and their orthologs, alleles, mutants, polymorphic variants, and conservatively modified variants combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (*see* U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of aging associated protein-encoding genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify homologs and orthologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of aging associated protein encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

[0099] Gene expression of aging associated proteins can also be analyzed by techniques known in the art, *e.g.*, reverse transcription and amplification of mRNA, isolation of total RNA or poly A^+ RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density polynucleotide array technology, *e.g.*, and the like.

[0100] Nucleic acids encoding aging associated proteins can be used with high density oligonucleotide array technology (*e.g.*, GeneChip™) to identify aging associated proteins, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs and orthologs being identified are linked to modulation of aging associated proteins, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al., AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al., Nat. Med.* 2:753-759 (1996); Matson *et al., Anal. Biochem.* 224:110-106 (1995); Lockhart *et al., Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al., Genome Res.* 8:435-448 (1998); Hacia *et al., Nucleic Acids Res.* 26:3865-3866 (1998).

[0101] The gene for aging associated proteins are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, *e.g.*, plasmids, or shuttle vectors.

EXPRESSION IN PROKARYOTES AND EUKARYOTES

[0102] To obtain high level expression of a cloned gene, such as those cDNAs encoding aging associated proteins, one typically subclones aging associated protein encoding nucleic acids into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook *et al.*, and Ausubel *et al, supra*. Bacterial expression systems for expressing aging associated proteins are available in, *e.g.*, *E. coli, Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

[0103] Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0104] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the

expression of aging associated protein encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding aging associated proteins and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0105] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0106] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[0107] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0108] Expression of proteins from eukaryotic vectors can be also be regulated using inducible promoters. With inducible promoters, expression levels are tied to the concentration of inducing agents, such as tetracycline or ecdysone, by the incorporation of response elements for these agents into the promoter. Generally, high level expression is obtained from inducible promoters only in the presence of the inducing agent; basal expression levels are minimal. Inducible expression vectors are often chosen if expression of the protein of interest is detrimental to eukaryotic cells.

[0109] Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with mitochondrial respiratory chain protein encoding sequences and glycolysis protein encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0110] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

[0111] Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of aging associated proteins, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

[0112] Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.*, Sambrook *et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing aging associated proteins.

[0113] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of aging associated proteins, which is recovered from the culture using standard techniques identified below.

[0114] Expression vectors with appropriate regulatory sequences can also be used to express a heterologous gene in a nematode. In one example, the expression vector is injected in the gonad of the nematode, and the vector is incorporated, *e.g.*, as an extra-chromosomal array in progeny of the nematode. The vector can further include a second gene (*e.g.*, a marker gene) that indicates the presence of the vector. For example, the heterologous gene can be a mammalian gene, *e.g.*, a mammalian cDNA, or a fragment thereof.

PURIFICATION OF AGING ASSOCIATED PROTEINS

[0115] Either naturally occurring or recombinant aging associated proteins can be purified for use in functional assays. Naturally occurring aging associated proteins can be purified, *e.g.*, from human tissue. Recombinant aging associated proteins can be purified from any suitable expression system.

[0116] Aging associated proteins may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*).

[0117] A number of procedures can be employed when recombinant aging associated proteins are being purified. For example, proteins having established molecular adhesion properties can be reversible fused to aging associated proteins. With the appropriate ligand, aging associated proteins can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, aging associated proteins could be purified using immunoaffinity columns.

A. Purification of aging associated proteins from recombinant bacteria

[0118] Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

[0119] Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of aging associated protein inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation

and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g.*, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

[0120] If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. Aging associated proteins are separated from other bacterial proteins by standard separation techniques, *e.g.*, with Ni-NTA agarose resin.

[0121] Alternatively, it is possible to purify aging associated proteins from bacteria periplasm. After lysis of the bacteria, when the aging associated proteins are exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM $MgSO_4$ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

B. Standard protein separation techniques for purifying aging associated proteins

Solubility fractionation

[0122] Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size differential filtration

[0123] The molecular weight of the aging associated proteins can be used to isolate it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column chromatography

[0124] The aging associated proteins can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins

immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

IMMUNOLOGICAL DETECTION OF AGING ASSOCIATED PROTEINS

[0125] In addition to the detection of aging associated genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect modulation of aging associated proteins. Such assays are useful for screening for modulators of aging associated proteins, *e.g.*, for regulation of lifespan, as well as for therapeutic and diagnostic applications. Immunoassays can be used to qualitatively or quantitatively analyze aging associated proteins. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

[0126] Methods of producing polyclonal and monoclonal antibodies that react specifically with the aging associated proteins are known to those of skill in the art (*see, e.g.*, Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.*, Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)).

[0127] A number of immunogens comprising portions of aging associated proteins may be used to produce antibodies specifically reactive with an aging associated protein. For example, recombinant protein or an antigenic fragment thereof, can be isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

[0128] Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (*e.g.*, BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see,* Harlow & Lane, *supra*).

[0129] Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see,* Kohler & Milstein, *Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, *et al.*, *Science* 246:1275-1281 (1989).

[0130] Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against non-specific proteins, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a K_d of at least about 0.1 mM, more usually at least about 1 μM, preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better. Antibodies specific only for a particular ortholog, such as a human ortholog, can also be made, by subtracting out other cross-reacting orthologs from a species such as a non-human mammal.

[0131] Once the specific antibodies against aging associated proteins are available, the protein can be detected by a variety of immunoassay methods. In addition, the antibody can be used therapeutically as aging associated protein modulators, *e.g.*, to enhance and extend

lifespan or to prevent premature aging. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay (Maggio, ed., 1980); and Harlow & Lane, *supra*.

[0132] It is also possible to use protein arrays to detect an aging associated protein, *e.g.*, to concurrently detect a plurality of aging associated proteins. Exemplary methods for producing protein arrays are provided in De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res. 28*, e3, I-VII; MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 0/98534, WO01/83827, WO02/12893, WO 00/63701, WO 01/40803 and WO 99/51773. In some implementations, polypeptides (including peptides) are spotted onto discrete addresses of the array, *e.g.*, at high speed, *e.g.*, using commercially available robotic apparati, *e.g.*, from Genetic MicroSystems or BioRobotics. The array substrate can be, for example, nitrocellulose, plastic, glass, *e.g.*, surface-modified glass. The array can also include a porous matrix, *e.g.*, acrylamide, agarose, or another polymer.

ASSAYS FOR MODULATION OF AGING ASSOCIATED PROTEINS

A. Assays

[0133] Modulation of aging associated proteins and genes can be assessed using a variety of *in vitro* and *in vivo* assays, as described herein, and, such assays can be used to identify inhibitors and activators of aging associated proteins. Such modulators of aging associated proteins and genes, which are involved in aging, are useful for enhancing lifespan or treating premature aging. Modulation of aging associated proteins and genes is tested using either recombinant or naturally occurring, preferably *C. elegans*, mouse, rat, guinea pig, monkey, or human aging associated proteins.

[0134] Preferably, the aging associated proteins or genes will have a *C. elegans* or a mammalian, *e.g.*, a rat, mouse, guinea pig, rabbit, monkey, or human sequence. Alternatively, the aging associated proteins or genes of the assay will be derived from a eukaryote and include an nucleic acid or amino acid subsequence having sequence identity to the *C. elegans* genes and gene products described herein. Generally, the sequence identity

will be at least 30%, 35%, 40%, 45% or 50%, preferably at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90%, most preferably at least 95%.

[0135] Measurement of modulation of aging phenotype with aging associated proteins or cells expressing aging associated proteins or genes, either recombinant or naturally occurring, can be performed using a variety of assays, *in vitro*, *in vivo*, and *ex vivo*. A suitable physiological change that affects activity can be used to assess measure modulation of aging associated genes. When the functional effects are determined using intact cells or animals, one can also measure a variety of effects such as, increases or decreases in lifespan, cellular proliferation, or in the case of signal transduction, hormone release, transcriptional changes to both known and uncharacterized genetic markers (*e.g.*, northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as cGMP.

[0136] In one embodiment, modulation of aging associated proteins or genes is assayed *in vivo* by screening in *C. elegans* or in a mammalian model system (cellular or animal) for changes in mean and median lifespan.

[0137] Some aging associated proteins have measurable enzymatic activity. Thus, enzymatic assays can be performed to detect modulation of the aging associated proteins. Enzymatic activity can encompass a chemical reaction carried out by a protein, as well as binding of substrates, cofactors, regulatory compounds, or ligands to the protein. It may also be useful to monitor other properties of the aging associated protein, *e.g.*, a structural property (*e.g.*, conformation, oligomerization state, stability, mobility, and the like) or a cellular property (*e.g.*, cellular localization, accessibility, clustering, and the like). The protein activity and binding capabilities assayed will depend on the aging associated protein.

[0138] The functional activities described herein do not represent all of the enzymatic activities that could be found in aging associated proteins. For example, some aging proteins could act to down regulate transcription of messenger RNA. Still other aging proteins may functional, *e.g.*, as a structural scaffold or adaptor protein, *e.g.*, they may or may not have an enzymatic activity.

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

[0139] The dsRNA administered according to the methods of the invention can be administered using any means known in the art and in conjunction with any pharmaceutically

acceptable carrier known to those of skill in the art. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (*e.g.*, nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989). Administration can be in any convenient manner, *e.g.*, by injection, oral administration, inhalation, transdermal application, or rectal administration.

[0140] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, *e.g.*, sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

[0141] The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0142] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for

example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of commends can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

[0143] Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for *ex vivo* therapy can also be administered intravenously or parenterally as described above.

[0144] The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient.

[0145] In determining the effective amount of the vector to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of the protein of choice, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

[0146] For administration, compounds and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

[0147] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

[0148] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1: RT-PCR analysis

[0149] Total RNA was extracted from approximately 20000 synchronized, sterile animals using trizol. To avoid mRNA contamination from eggs, which may not be susceptible to bacterial RNAi, we determined the efficacy of our RNAi treatments using a sterile strain, *fer-15(b26), fem-1(hc17)*. Before harvest, animals were exposed to bacteria containing the RNAi vector or containing the *daf-2* RNAi construct from the L1 until the L4 larval stage or from day 8 until day 10 of adulthood. 4 ug of total RNA was used for one round of reverse transcription (RT) using oligo dT primers. Serial dilutions of the RT reaction (1:1 – 1:24) was used for PCR reaction using *daf-2* specific primers (5'-GGCACCGGTGCGGGAGCATTGAAACGAACAAAACACATC-3', 5'-TCCAGCACATTTTCATCACCTTATACC-3') to the 3' end of *daf-2* . RNAi was directed to a non-overlapping 5' end of *daf-2*. Serial dilutions of the RT reaction (1:1 – 1:20) was used for PCR reaction using *daf-16* specific primers (5'-ATCTATGATGATCTAGAATTCCCATCATGGG -3', 5'-CAAATCAAAATGAATATGCTGCCCTCCAGC -3') to the 3' end of *daf-16*. RNAi was directed to a non-overlapping 5' end of *daf-16*. 4µl of a 50µl PCR reaction was analyzed on agarose gels using ethidium bromide.

Example 2: Dauer assays

[0150] Wild-type hermaphrodites were allowed to lay eggs onto the control RNAi bacteria or *daf-2* RNAi bacteria at 20°C. The eggs were then shifted to 27°C and the presence of dauer larvae were scored 48 hours later when animals would normally be reproductive adults.

Example 3: Lifespan, reproduction and stress assays

[0151] Lifespan, reproduction and stress assays were conducted at 20°C. We used the pre-fertile period of adulthood as the t=0 for lifespan analysis. Strains were grown at 20°C at for at least two generations before use in lifespan analysis. We used Statview 5.0.1 (SAS)

software for statistical analysis and to determine means and percentiles. In all cases, P values were calculated using the logrank (Mantel-Cox) method.

[0152] The total number of progeny born to a single worm over time was measured in the following way. Briefly, worms hatched within a 1 hour period were collected and allowed to develop to the L4 stage. Once in the L4 stage, worms were individually placed onto separate plates. In all cases, at least 15 worms were used for each analysis. Worms were transferred to new plates every 12 hours and the resulting progeny were allowed to grow for two days until counted for progeny measurements. The % of total progeny was calculated for each time point by dividing the number of progeny produced on a time point by the total number of progeny produced over the course of the experiment.

[0153] For stress resistance assays, wild-type animals were transferred to bacteria expressing *daf-2* dsRNA at the indicated times. After reproduction had ceased, at day 5 of adulthood, worms were submerged in 50µl of 0.4M paraquat dissolved in S-basal buffer at 20 °C. Death was determined on an hourly basis by the lack of movement after prodding with a platinum wire. At least 40 worms were used for each analysis.

Example 4: RNAi inhibition using *dcr-1*

[0154] To lower *daf-2* activity during the larval stages only, wild-type animals were grown on bacteria expressing *daf-2* dsRNA and then shifted to bacteria expressing *dcr-1* dsRNA as day 1 adults. Control animals were grown during development on the RNAi bacteria containing the vector only and then shifted to *dcr-1* RNAi bacteria as day 1 adults. Animals were grown at 25°C.

[0155] These experiments demonstrate that a component of the RNAi response such as dicer dsRNA can conveniently be used to block an existing RNAi response. In the first, loss of daf-2 activity will increase the lifespan of C. elegans if inactivated during early adulthood. Daf-2 RNA was inactivated using daf-2 specific RNAi. The animals were removed from the environmental RNAi stimulus (food bacteria expressing daf-2 dsRNA). The RNAi response continued to exert its effect during the adult stages and caused an increased lifespan. By shifting these animals to dcr-1 RNAi in early adulthood, increased lifespan was blocked, by blocking the existing RNAi response against daf-2 (see Figure 5).

[0156] In the second experiment loss of mitochondrial electron transport activity during the early developmental stages causes an increased adult lifespan. In contrast to the daf-2

experiment, this increased lifespan could not be reduced if the animals were shifted to dcr-1 RNAi as adults. Loss of dcr-1 function does not merely shorten lifespan (see Figure 6).

[0157] Finally, wild-type worms treated with dcr-1 RNAi have normal lifespans, further indicating that dcr-1 does not function by merely reducing lifespan.

[0158] The nucleic acid sequence for C. elegans dcr-1 is K12H4.8 (WormBase) and the protein sequence is WP:CE25057 (WormBase). The GenBank Accession No. for C. elegans dcr-1 is NM_066360.1 (nucleic acid, SEQ ID NO:1) and NP_498761.1 (protein, SEQ ID NO:2). The GenBank Accession No. for human dcr-1 is NM_030621.1 (nucleic acid, SEQ ID NO:3) and NP_085124.1 (protein, SEQ ID NO:4).

[0159] Insulin/IGF-1 signaling influences longevity, reproduction and diapause in many organisms. Because of the fundamental importance of this system in animal physiology, we asked when during the animal's life it is required to regulate these different processes. We find that in *C. elegans,* the pathway acts during adulthood, to relatively advanced ages, to influence aging. In contrast, it acts during development to regulate diapause. In addition, the pathway controls longevity and reproduction independently of one another. Together our findings show that lifespan regulation can be dissociated temporally from phenotypes that might seem to decrease the quality of life.

[0160] In *C. elegans,* mutations that decrease the activity of DAF-2, an insulin/IGF-1-like receptor, or downstream PI-3 kinase/PDK-1/AKT signaling components, prolong youthfulness and double the lifespan of the animal. The DAF-2 pathway influences other processes as well. All *daf-2* mutations examined increase resistance to oxidative stress and delay reproduction (some alleles also reduce fertility). Strong *daf-2* mutations cause juvenile animals to enter a state of diapause, called dauer, instead of growing to adulthood. All of these mutant phenotypes require the activity of DAF-16, a forkhead family transcription factor (*1-3*).

[0161] Mutations in components of the insulin/IGF-1 pathway also extend lifespan in flies (*4, 5*), and mutations that inhibit pituitary development or growth hormone receptor signaling, which in turn decrease IGF-1 signaling, extend the lifespan of mice (*6*). Components of insulin/IGF-1 signaling pathways also influence reproduction, stress resistance and entry into diapause-like states in a wide range of organisms (*1-3*).

[0162] To investigate when the *C. elegans* insulin/IGF-1 pathway acts to regulate longevity, diapause, reproduction and stress resistance, we used RNAi (RNA interference), which decreases mRNA levels (*7*), to decrease *daf-2* and *daf-16* activity at different times during the life cycle. RT-PCR analysis confirmed that feeding animals bacteria expressing *daf-2* or *daf-16* dsRNA decreased mRNA levels (Fig. 1) (*8*). We found that culturing wild-type animals on bacteria expressing *daf-2* dsRNA from the time of hatching produced Daf-2(-) phenotypes similar to those produced by partial loss-of-function *daf-2* mutations (*9-12*). At 20° the animals grew to adulthood and became long-lived, stress-resistant adults with normal brood sizes but protracted reproductive schedules (Fig. 2A; Tables 1 and 2) (*8*). High temperature is known to induce some dauer formation in wild type (*13*). Our *daf-2* RNAi treatment increased the frequency of dauer formation at high temperatures [27°C; 45% dauers (n = 72) vs. 10% dauers (n = 81) for control animals] (*8*). Strong *daf-2* mutations delay growth to adulthood, and cause uncoordinated movement and some embryonic lethality (*9, 14, 15*). We did not observe these phenotypes (not shown), suggesting that they result from greater reduction of *daf-2* activity. Conversely, feeding *daf-2(1370)* mutants bacteria expressing *daf-16* dsRNA completely suppressed their delayed development (*16*), longevity and reproductive phenotypes (Fig. 3A). This *daf-2* mutation is temperature sensitive. At 25.5°, the animals become dauers; this phenotype was partially suppressed by *daf-16* RNAi (*17*).

[0163] To ask when *daf-2* influences adult longevity, we shifted wild-type animals to bacteria expressing *daf-2* dsRNA at different ages and determined their lifespans. We found that initiating *daf-2* RNAi treatment in young adults extended lifespan to the same extent as did initiating RNAi at hatching (Fig. 2A vs. 2G, P=0.38, Table 1). Thus *daf-2* is required during adulthood to regulate adult lifespan.

[0164] To determine whether *daf-2* could also function during development to influence adult lifespan, we lowered insulin/IGF-1 signaling specifically during development. First, we initiated *daf-2* RNAi during development and then attempted to turn off the RNAi process during adulthood (*8*). We reasoned that if animals were exposed to dsRNA of a gene required for RNAi to function, such as *dcr-1* (dicer) (*18*), then RNAi activity should remain low. To this end, we shifted animals exposed to *daf-2* RNAi at hatching onto bacteria expressing *dcr-1* dsRNA when they reached adulthood. These animals did not have long lifespans (Fig. 4, Table 1).

[0165] We also turned off the effects of a *daf-2* mutation at different stages by shifting *daf-2(e1370)* mutants to bacteria expressing *daf-16* dsRNA. We found that initiating *daf-16* RNAi treatment during young adulthood completely suppressed the long lifespans of *daf-2(e1370)* mutants (Fig. 3A vs. 3G, p=0.19, Table 1). Thus the presence of DAF-16 activity during development was irrelevant. Therefore, the insulin/IGF-1 pathway appears to operate exclusively during adulthood to influence adult lifespan.

[0166] We found that initiating *daf-2* RNAi treatment later in adulthood, throughout the reproductive period, also extended lifespan (Fig. 2, G-J, Table 1). The magnitude of this extension declined steadily, and became insignificant after about 6 days of adulthood (Fig. 2M, and Table 1), though there was a sharp decline in *daf-2* mRNA at this time (Fig. 1A). Treating *daf-2(e1370)* mutants with *daf-16* RNAi during the reproductive period also extended lifespan (Fig. 3, H-L, Table 1). Again, the magnitude of the effect declined with age, although we continued to observe effects on lifespan until about day 15 of adulthood (Fig. 3, M-P, Table 1). In these experiments, *daf-2* and *daf-16* appeared to function for different durations during adulthood; however, wild-type animals (used in the *daf-2* RNAi experiments) age more quickly than *daf-2* mutants (used in the *daf-16* RNAi experiments). In fact, as assayed by tissue morphology, day 6 wild-type animals are approximately the same age as day 15 *daf-2(e1370)* mutants (*19*). Thus, both genes may function at the same time, throughout the reproductive period, to influence aging. We note that the pathway could conceivably function later as well (if, for example, the rate of protein turnover falls in old animals).

[0167] We next investigated when *daf-2* and *daf-16* RNAi could affect the timing of reproduction. Surprisingly, treating adults with *daf-2* RNAi had no effect on reproduction (Fig. 2, F-J, and Table 1). Likewise, treating *daf-2* mutants with *daf-16* RNAi during adulthood failed to suppress the *daf-2* reproductive phenotype (Fig. 3, G-L, and Table 1). In contrast, initiating either RNAi treatment at hatching did affect the timing of reproduction (Fig. 2A and Fig. 3A). Thus *daf-2* appears to control reproduction and longevity independently from one another.

[0168] To determine when *daf-2* and *daf-16* might function in reproduction, we subjected the animals to RNAi during development. We found that initiating *daf-2* RNAi treatment of wild-type animals at any time before the last larval stage, L4, delayed reproduction (Fig. 2, A-D), and that initiating *daf-16* RNAi treatment of *daf-2(e1370)* mutants at any time before

L4 restored normal reproductive timing (Fig. 3, A-E). Treatment during or after L4 did not affect reproduction (Fig. 2, E-J and Fig. 3, F-L). These findings show that reproductive timing can be specified independently of the dauer decision (which occurs prior to L3) (*13*), and they suggest that the *daf-2* pathway may function late in development to affect the timing of reproduction. However, we cannot rule out the possibility that the DAF-2 pathway controls reproduction during adulthood, but that initiating RNAi during or after L4 does not lower signaling activity below threshold until after DAF-2 and DAF-16 have completed their roles in reproduction. This seems less likely, because message levels fall sharply within 48 hours of RNAi treatment (Fig. 1), whereas reproduction continues for many days.

[0169] The DAF-2 pathway also regulates stress resistance (*9-11, 20*). Because *daf-2* larvae (*21*), and dauers (*13*), are stress resistant, *daf-2* must regulate stress resistance in the larvae. We found that animals treated with *daf-2* dsRNA as adults were resistant to the oxidative-damaging agent paraquat (Table 2). Thus, *daf-2* also acts in the adult to regulate stress resistance. Since the DAF-2 pathway regulates lifespan during adulthood as well, this finding supports the hypothesis that increased resistance to oxidative stress contributes to longevity (*9-11, 20*). Consistent with this, overexpression of the antioxidant superoxide dismutase gene during adulthood can extend the lifespan of *Drosophila* (*22, 23*), as can administering the antioxidant Euk134 to adult worms (*24*).

[0170] In summary, in this study, we used RNAi to reduce *daf-2* and *daf-16* activity at specific times during the life cycle. As with any conditional expression system, we cannot be certain when any residual *daf-2* or *daf-16* activity, not removed by RNAi, might function. However, the fact that *daf-16* RNAi completely suppressed the strong longevity and reproductive phenotypes of the *daf-2(e1370)* mutant suggests that the level of residual pathway activity is likely to be minimal, as does the fact that inferences from reciprocal *daf-2* and *daf-16* RNAi experiments were always in agreement.

[0171] Our findings suggest that the DAF-2 pathway functions exclusively during adulthood, throughout the reproductive period, to influence adult lifespan. This suggests that the pathway controls downstream gene expression in an ongoing fashion through much of adulthood. Because the dauer is a juvenile form (*25*), the DAF-2 pathway must act in separate regulatory events to control dauer formation and adult lifespan. Previously we proposed that *daf-2* regulates a longevity process that determines the lifespan of adults but that can also be expressed in conjunction with dauer-specific traits to give dauers their long

lifespans (*12*). It will be interesting to learn whether the pathway regulates the same genes at two different life stages to influence the lifespans of adults and dauers.

[0172] Our findings indicate that the DAF-2 pathway participates in multiple, independent regulatory events to influence aging, reproduction and diapause. In this regard, the pathway is similar to many growth factor signaling pathways (such as the EGF or TGF-beta pathways), which regulate different aspects of cell growth and differentiation independently of one another. The pleiotropy of insulin/IGF-1 pathway mutations in many organisms, particularly the linkage with diapause-like states, has raised the possibility that longevity achieved through this pathway would invariably be associated with impaired growth or reproduction. Instead, our findings suggest that, in other organisms as well, it may be possible to manipulate insulin/IGF-1 signaling during adulthood so as to extend youthfulness and lifespan without affecting either of these processes.

Table 1. Effects of *daf-2* RNAi and *daf-16* RNAi on lifespan and brood size.

Treatment	Mean Lifespan ± s.e.m. (days)	*p* †	75th Percentile* (days)	Average Brood Size ± SD	(Total #Animals Died/Total)§
Egg	35.5 ± 1.9	<0.0001 ‡	48	339 ± 41	61/81
L1	35.4 ± 2.6	<0.0001 ‡	52	354 ± 42	33/52
L2	36.2 ± 2.3	<0.0001 ‡	48	373 ± 43	41/48
L3	34.6 ± 1.9	<0.0001 ‡	48	380 ± 44	52/74
L4	35.3 ± 1.9	<0.0001 ‡	48	340 ± 47	56/71
Pre-Fertile Adult	29.1 ± 1.3	<0.0001 ‡	31	344 ± 46	56/71
Day 1 Adult	34.3 ± 2.0	<0.0001 ‡	45	386 ± 88	40/53
Day 2 Adult	29.9 ± 2.3	<0.0001 ‡	41	361 ± 53	36/37
Day 3 Adult	26.6 ± 1.8	0.0006 ‡	35	341 ± 50	42/52
Day 4 Adult	26.9 ± 1.8	0.0001 ‡	33	354 ± 53	33/39
Day 5 Adult	23.7 ± 1.0	0.0051 ‡	28	N.D.	27/35
Day 6 Adult	22.3 ± 1.4	0.0072 ‡	28	N.D.	35/44
Day 8 Adult	19.0 ± 0.8	0.5719 ‡	22	N.D.	49/53
Vector(control)	19.7 ± 0.8		22	371 ± 57	46/53
***daf-2(e1370)* shifted to *daf-16* RNAi as:**					
Egg (α)	17.9 ± 0.6	<0.0001 Ø	21	269 ± 39	35/39
Egg (β)	19.1 ± 0.8	<0.0001¥	25	N.D.	32/47
L1	17.8 ± 0.4	<0.0001 Ø 0.8509	21	262 ± 37	74/86

L2	17.8 ± 0.4	<0.0001 Ø 0.7903	21	295 ± 35	67/73
L2/L2d	17.1 ± 0.3	<0.0001 Ø 0.8995	20	312 ± 42	67/72
L2d/L3	17.2 ± 0.5	<0.0001 Ø 0.5973	20	315 ± 43	67/69
L4	18.6 ± 0.4	<0.0001 Ø 0.1954	22	318 ± 41	67/80
Pre-Fertile Adult	20.3 ± 0.4	<0.0001 Ø 0.0006	22	310 ± 29	71/73
Day 1 Adult	22.2 ± 0.5	<0.0001 Ø <0.0001	25	278 ± 26	62/67
Day 2 Adult	20.7 ± 0.5	<0.0001 Ø 0.0005	25	270 ± 23	73/76
Day 3 Adult	21.9 ± 0.5	<0.0001 Ø <0.0001	25	253 ± 20	75/85
Day 4 Adult	24.3 ± 0.6	<0.0001 Ø <0.0001	27	271 ± 20	76/83
Day 5 Adult	25.6 ± 1.0	<0.0001 Ø <0.0001	29	255 ± 20	47/63
Day 6 Adult	24.2 ± 0.7	<0.0001 Ø <0.0001	28	N.D.	69/73
Day 7 Adult	24.5 ± 0.7	<0.0001 Ø <0.0001	27	N.D.	67/85
Day 10 Adult	28.9 ± 1.0	<0.0001¥ <0.0001 <0.0001ÿ	33	N.D.	44/51
Day 15 Adult	33.9 ± 1.6	<0.0001¥ <0.0001 <0.0001ÿ	38	N.D.	40/47
Day 23 Adult	41.5 ± 2.3	0.0498¥ <0.0001 0.0951ÿ	51	N.D.	39/55
Day 30 Adult	44.6 ± 2.2	0.2344¥ <0.0001 0.4830ÿ	54	N.D.	45/56
Day 40 Adult	49.6 ± 2.7	0.4305¥ <0.0001 0.3095ÿ	56	N.D.	43/53
Vector (α)	41.2 ± 1.7	<0.0001	50	263 ± 21	59/74
Vector (β)	47.3 ± 2.5	<0.0001	59	N.D.	43/57
Repression of *daf-2* RNAi by *dcr-1* dsRNA:					

N2 grown on RNAi bacteria during development, then shifted to *dcr-1* RNAi bacteria	12.5 ± 0.5	N.D.	14	N.D.	54/60
N2 grown on *daf-2* RNAi bacteria during development, then shifted to *dcr-1* RNAi bacteria	13.7 ± 0.6	0.0417æ	17	N.D.	45/50
N2 grown on *daf-2* RNAi bacteria during development and adulthood	28.8 ± 1.1	<0.0001 æ	33	N.D.	50/50

[0173] * The 75th percentile is the age when the fraction of animals alive reaches 0.25.

[0174] † *P* values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time.

[0175] § The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The logrank (Mantel-Cox) test was for statistical analysis.

[0176] Average brood size was calculated from the total brood size of at least 15 animals cultured independently in each trial.

[0177] ‡ Compared with N2 worms grown on HT115 bacteria harboring the RNAi plasmid vector at 20°C.

[0178] Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi plasmid Egg (α), at 20 °C, which were analyzed at the same time.

[0179] Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi plasmid, Egg (β), at 20°C, which were analyzed at the same time.

[0180] Ø Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid, Vector (α), at 20°C, which were analyzed at the same time.

[0181] ¥ Animals containing vector only (β), at 20°C, which were analyzed at the same time, were compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid.

[0182] ÿ *p* value after resetting T_0 of lifespans to time at which RNAi treatment was initiated. For instance; T_0 was set to 10 for assays in which the experimental population was treated with RNAi at day 10. Animals containing vector only (β), at 20°C, were compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid.

[0183] æ Compared with N2 worms grown on HT115 bacteria harboring the RNAi plasmid vector during development, then shifted to bacteria expressing *dcr-1* dsRNA at 25°C.

Table 2. Effect of *daf-2* function on stress resistance.

	% Alive in 0.4M paraquat after:					
	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
Control (RNAi vector bacteria)	*52 #56	48 50	32 34	20 22	4 12	0 4
daf-2 RNAi initiated at hatching	*78 #87	76 83	74 70	47 52	44 40	41 38
daf-2 RNAi initiated as day-1 adult	*94 #90	94 85	72 70	56 58	53 44	50 34

The results of two independent trials are shown (n ÿ 25 trial 1, n ÿ 40 trial 2).
*Trial 1 is the first row of numbers.
Trial 2 is the second row of numbers.

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